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*J Immunol* 2018; 200:3599-3611; Prepublished online 9 April 2018; doi: 10.4049/jimmunol.1701552
http://www.jimmunol.org/content/200/10/3599

Supplementary Material http://www.jimmunol.org/content/suppl/2018/04/07/jimmunol.1701552.DCSupplemental

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
The Synergy between Palmitate and TNF-α for CCL2 Production Is Dependent on the TRIF/IRF3 Pathway: Implications for Metabolic Inflammation

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The chemokine CCL2 (also known as MCP-1) is a key regulator of monocyte infiltration into adipose tissue, which plays a central role in the pathophysiology of obesity-associated inflammation and insulin resistance. It remains unclear how CCL2 production is upregulated in obese humans and rodents. Because elevated levels of the free fatty acid (FFA) palmitate and TNF-α have been reported in obesity, we studied whether these agents interact to trigger CCL2 production. Our data show that treatment of THP-1 and primary human mononuclear cells with palmitate and TNF-α led to a marked increase in CCL2 production compared with either treatment alone. Mechanistically, we found that cooperative production of CCL2 by palmitate and TNF-α did not require MyD88, but it was attenuated by blocking TLR4 or TRIF. IRF3-deficient cells did not show synergistic CCL2 production in response to palmitate/TNF-α. Moreover, IRF3 activation by polyinosinic-polycytidylic acid augmented TNF-α–induced CCL2 secretion. Interestingly, elevated NF-κB/AP-1 activity resulting from palmitate/TNF-α costimulation was attenuated by TRIF/IRF3 inhibition. Diet-induced C57BL/6 obese mice with high FFAs levels showed a strong correlation between TNF-α and CCL2 in plasma and adipose tissue and, as expected, also showed increased adipose tissue macrophage accumulation compared with lean mice. Similar results were observed in the adipose tissue samples from obese humans. Overall, our findings support a model in which elevated FFAs in obesity create a milieu for TNF-α to trigger CCL2 production via the TLR4/TRIF/IRF3 signaling cascade, representing a potential contribution of FFAs to metabolic inflammation. *The Journal of Immunology, 2018, 200: 3599–3611.

Obesity-mediated low-grade chronic inflammation plays a key role in the development of various diseases, including type 2 diabetes, atherosclerosis, and hepatic steatosis. In obesity, adipose tissue is dysregulated by the infiltration of immune cells, associated with increased production of cytokines and chemokines. In particular, proinflammatory macrophage accumulation in adipose tissue is an important feature of obesity, correlating with local expression of inflammatory markers, including TNF-α, IL-6, IL-1β, and MCP-1 (encoded by the Cc2 gene) (1, 2). These inflammatory mediators, especially CCL2 (MCP-1), contribute to the infiltration of circulatory monocytes into the adipose tissue that play a central role in the development and maintenance of obesity-associated chronic low-grade inflammation (3, 4).

Metabolic inflammation is considered instrumental in the development of insulin resistance. In obese mice and humans, adipose tissue mRNA, as well as circulating CCL2 levels, correlate positively with the degree of corpulence and insulin resistance. CCL2- and CCR2-deficient mice show reduced adipose tissue macrophages, lower levels of inflammatory mediators, and improved insulin sensitivity on a high-fat diet (HFD) (3, 5). CCL2 is produced by monocytes/macrophages, fibroblasts, astrocytes, endothelial/epithelial cells, smooth muscle cells, mesangial cells, adipocytes, and microglial cells. Among these cell types, monocytes/macrophages are the predominant source of CCL2 (6), which is secreted in response to various proinflammatory stimuli, including TNF-α (7), IL-1β (8), LPS (9), and free fatty acids (FFAs) (9, 10). Notably, the mechanism(s) triggering abnormally high CCL2 levels in obesity remain unclear.

Among the many factors promoting obesity-associated inflammation, we have been interested specifically in the roles of FFAs design of some experiments; J.T. critically reviewed the manuscript and provided technical feedback; S.S. participated in data analysis and the writing of the manuscript; M.K. performed all mice-related experiments; and E.D.R. supervised all technical feedback; S.S. participated in data analysis and the writing of the manuscript; Y.A.H. participated in the online version of this article contains supplemental material. Abbreviations used in this article: BMI, body mass index; CPZ, chlorpromazine; DIO, HFD-induced obese; FFA, free fatty acid; HFD, high-fat diet; IHC, immunohistochemistry; OW, overweight; pol Ic, polyinosinic-polycytidylic acid; Rfu, relative fluorescence unit; SEAP, secreted embryonic alkaline phosphatase; siRNA, small interfering RNA; WAT, white adipose tissue. This article is distributed under The American Association of Immunologists, Inc., Reuse Terms and Conditions for Author Choice articles.
and TNF-α. In obesity and type 2 diabetes, plasma and tissue levels of FFAs are elevated and have been suggested to contribute to the development of chronic inflammation and insulin resistance (11). FFAs can activate inflammatory signaling pathways through various mechanisms, including activation of the innate immune receptor TLR4 (12). TLR4 is a member of the family of pattern-recognition receptors that play a key role in innate immunity by activating proinflammatory signaling pathways in response to microbial pathogens (e.g., LPS) and damaged tissue-associated signals or alarms, such as high-mobility group box 1 protein. LPS binding to TLR4 activates two intracellular signaling pathways, one of which requires the adaptor protein MyD88, whereas the other uses the adaptor TRIF. MyD88 triggers a downstream signaling cascade, leading to the activation of NF-κB and MAPK pathways. TRIF associates with TLR4 after ligand-induced internalization and leads to IRF3 activation and its downstream signaling cascade (13).

In the obese state, FFA-mediated inflammation enhances the polarization of classically activated M1-type inflammatory macrophages in the white adipose tissue (WAT), which secrete proinflammatory cytokines, including TNF-α, IL-1β, and IL-6 (14). Of note, several reports show that elevated TNF-α levels in obesity correlate positively with insulin resistance (15, 16). Deletion of TNF-α or its receptors was shown to improve insulin sensitivity in obese animals, with reduced macrophage infiltration into adipose tissue. Several studies have documented consistently higher levels of CCL2, FFAs, and TNF-α in the circulation of obese/type 2 diabetes individuals; therefore, we asked whether FFAs and TNF-α could cooperate in triggering CCL2 (MCP1) production in monocytic cells. In this article, we present data showing that long-chain fatty acids, including palmitate, exert multiplicative effects on CCL2 expression in monocytic cells when coadministered with TNF-α. We further show that TLR4 is required for this cooperative and synergistic or additive activity and, specifically, that the TRIF/IRF3 (i.e., MyD88-independent) arm of the TLR4 signaling pathway is critical for this effect. Pointing to the clinical significance of this synergy, our murine and human data show a remarkable association between TNF-α and CCL2 levels in obese mice and humans.

Materials and Methods

Reagents, Abs, and cell lines

Palmitate (catalog number P5585), oleate (catalog number O1008), myristate (catalog number M3128), laurate (catalog number w261416), sodium butyrate (catalog number B5887), LPS (catalog number L4391), Trolox (catalog number 238813), NDGA (catalog number 74540), PMA (catalog number P1585), polysinosinic-polycytidylic acid (poly I:C; catalog number P9582), chlorpromazine (catalog number C8138), and resveratrol (catalog number R5010) were purchased from Sigma (San Diego, CA). Recombinant human TNF-α (catalog number 210-1A-100) and recombinant human MCP-1 (catalog number 279-MC-010) were obtained from R&D Systems (Minneapolis, MN). Pepinh-TRIF (trif inhibitory peptide: RQIKIWFQNRRMKWKK-FCEEFQVPGRGELH-NH2; Pepinh-Control: RQIKIWFQNNRMKWKK-FCYESQVPGRGELH-NH2; Pepinh-Control: RQIKIWFQSNRMKWKK-SILHRGPDMEAFFI-NH2; catalog number tlr-pitrif), Bay11-7085 (catalog number B5565), and QUANTI-Blue medium (catalog number rep-qb-2) were purchased from InvivoGen. THP-1 cells (ATCC TIB-202, 2 × 10⁶ cells/ml), were purchased from InvivoGen. Human TNF-R1/ TNFRSF1A mAb (catalog number MAB6256) and mouse IgG1 (MAB002R) were purchased from R&D Systems. IRF3-1 (DSB9) Rabbit mAb (catalog number 4302), Phospho-IRF3-1 (Ser396) (catalog number 4947), and β-actin Ab (catalog number 4967) were purchased from Cell Signaling Technology. Anti-MCP1 Ab (ab9669) and Goat Anti-Rabbit IgG H&L (Alexa Fluor 488) (ab150077) were purchased from Abcam (Cambridge, MA). FITC-conjugated anti-TLR4 mAb (catalog number IMG-417C) was obtained from Novus Biologicals. FITC-conjugated mouse isotype Ab (catalog number 555742) and Anti-Human CD120a Ab (catalog number 550514) were purchased from BD Biosciences (San Jose, CA).

The human monogenic leukemia THP-1 cell line was purchased from American Type Culture Collection. THP1-XBlue cells stably expressing a secreted embryonic alkaline phosphatase (SEAP) reporter inducible by NF-κB and AP-1, as well as THP1-XBlue-defMyD cells deficient in MyD88 activity (MyD88−/−/THP-1 cells), were purchased from InvivoGen.

Cell culturing and macrophage preparation

THP-1 and primary monocytes (1 × 10⁶ cells per milliliter) were cultured using 12-well plates (Costar, Corning Incorporated, Corning, NY) in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% FBS (Life Technologies), 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100 μg/ml Normocin, 50 μM penicillin, and 50 μg/ml streptomycin, and were incubated at 37°C in 5% CO₂ under humidity. THP1-XBlue cells were cultured in complete RPMI medium containing Zeocin (200 μg/ml) to select for cells expressing the SEAP–NF-κB/AP-1 reporter. THP1-XBlue-defMyD cells were cultured in complete RPMI medium containing Zeocin (200 μg/ml) and HydroGold (100 μg/ml). THP-1 cells were differentiated into macrophages by treatment with PMA (10 ng/ml) for 3 d in routine culturing media.

Mice

Male C57BL/6 mice were obtained from RIKEN BRC Experimental Animal Division. Animal studies were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines. Control (lean) mice were fed a standard chow diet (8664; Harlan Teklad; 6.4% w/w fat) and maintained under a regular 12/12-h light/dark cycle at a room temperature of 23°C. Mice fed an HFD were maintained on Research Diets 12331i (42% kcal from fat) and housed under identical light/dark cycle and temperature conditions as control mice for a period of 16 wk. Body weights were measured weekly. Inguinal WAT samples were collected, flash-frozen in liquid nitrogen, and stored at −80°C until use. Mouse body mass composition was examined by magnetic resonance imaging (Echo Medical Systems). Blood samples were collected in EDTA tubes, from animals that had fasted for 14 h, and were centrifuged at 14,000 rpm for 10 min; plasma was aliquoted and stored at −80°C until use.

Plasma analyses

Plasma FFAs were measured using a commercial kit (Abcam). Plasma cytokines/chemokines were measured using a MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (catalog MCYT-MAG-70K-PPX3; EMD Millipore, Bedford, MA), following the manufacturer’s instructions. Data were acquired using a Luminex MILLIPLEX analyzer, and a digital processor managed data output. MILLIPLEX analyst software was used to determine fluorescence intensity and analyte concentrations (pg/ml).

Human samples

Subjects (26 lean/overweight [OW] and 26 obese) were recruited for the study at the Dasman clinics. All participants gave written informed consent, and studies were approved by the ethics committee of Dasman Diabetes Institute. The clinicodemographic data of the study participants are summarized in Supplemental Table I. Anthropometric and clinical measurements (including height and weight of the individuals) were taken using calibrated portable electronic scales and portable inflexible height-measuring bar. Blood was collected after an overnight fast (minimum 8 h) and analyzed for fasting glucose, glycated hemoglobin (HbA1c), and lipid profile using standard clinical laboratory procedures. Human adipose tissue samples (~0.5 g) were collected via abdominal s.c. fat pad biopsy lateral to the umbilicus, using standard surgical methods (17). Freshly collected adipose tissue samples (~50–100 mg) were preserved in RNA later and stored at −80°C until use. Adipose tissue total RNA was purified using an RNasy Kit, as per the manufacturer’s instructions.

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**PBMCs collection and monocyte purification**

Human peripheral blood samples (25–30 ml) were collected from healthy donors in EDTA Vacutainer tubes through venipuncture at Dasman Diabetics Institute, and PBMCs were isolated using a Ficoll-Paque density gradient method, as described (18, 19). Monocytes were purified from PBMCs using magnetic cell sorting method using human monocyte isolation kit II human; order number 130-091-153; Miltenyi Biotec) in which nonmonocytic cells, including T cells, NK cells, B cells, dendritic cells, and basophils, were magnetically labeled with a mixture of biotin-conjugated Abs against CD3, CD7, CD16, CD19, CD56, CD123, and CD235a (glycophorin A) surface Ags and anti-biotin MicroBeads. Purified CD14+ monocytes were eluted through magnetic column purification, and purity (>90%) was determined by flow cytometry.

**Cell stimulation and chemokine/cytokine measurements**

THP-1, primary human monocytes, or THP-1-XBlue cells were plated at a concentration of 1 × 10⁶ cells per well in 12-well plates. Cells were stimulated with palmitate (200 μM; Sigma) and TNF-α (10 ng/ml), alone or in combination, for 24 h at 37°C. THP-1 cells were also treated with IL-1β (10 ng/ml) or palmitate (200 μM), or both, for 24 h. Cells were harvested for RNA isolation, and conditioned media were collected and stored at −80°C until use for determining CCL2 production or SEAP activity. CCL2 secreted protein was measured in cell supernatants using a Human DuoSet Elisa Kit (R&D systems), following the manufacturer’s instructions.

**Immunocytofluorescence**

Following treatment, as indicated above, 1 × 10⁶ monocytic cells were washed in PBS and coated on slides using a cytospin technique at 600 rpm for 3 min. The slides were fixed in 4% formaldehyde and washed three times in cold PBS. Cells were permeabilized with 0.1% Triton X-100, washed three times in cold PBS, blocked in 1% BSA for 1 h, and incubated overnight with primary Ab (1:200 rabbit anti-human CCL2/MCP-1 polyclonal Ab; ab9669; Abcam) at room temperature. Cells were washed three times in PBS with 0.05% Tween and incubated with secondary Ab (Alexa Fluor 488 conjugated; ab150077; Abcam) for 1 h. After several washes in PBS, cells were counterstained and mounted using VECTASHIELD HardSet Antifade Mounting Medium with DAPI (catalog number H-1500; Vector Laboratories). Confocal images were collected using a Plan-Apochromat 63×1.40 oil DIC M27 objective lens (Inverted Zeiss LSM 710 Axio Observer microscopy; Gottingen, Germany), with excitation via a 590-nm diode-pumped solid-state laser and 405-nm line of an argon ion laser, and optimized emission detection bandwidths were configured using Zeiss ZEN 2010 software.

**Cell-migration assay**

Cell-migration assays were performed using a QCM Chemotaxis Cell Migration Assay, 96-well (5 μm) (Millipore), following the manufacturer’s instructions. Briefly, 1 × 10⁶ cells in serum-free medium were seeded into the migration chamber, and the lower chamber received conditioned media from THP-1 cells treated for 24 h with palmitate and TNF-α, individually or in combination, as well as conditioned media from untreated controls. Recombinant human CCL2/MCP-1 (2000 pg/ml) in serum-free medium was used in the lower chamber as a positive control. After incubation for 6 h, cells that had migrated through the 5-μm-pore membrane were detached and treated with the lysis buffer/dye solution provided. Sample fluorescence (OD), expressed as relative fluorescence units (RFU), was measured using a fluorescence microplate reader with an excitation of 480 nm and an emission of 520 nm. A standard curve (different cell concentrations and their RFU values) was plotted and used to calculate the number of cells that migrated based on the RFU values of test samples.

**Flow cytometry**

Following treatment as indicated, 1 × 10⁶ cells were washed in cold PBS and incubated with FITC-conjugated anti-TLR4 mAb (Novus Biologicals) and FITC-conjugated mouse IgG2b isotype Ab (BD Biosciences). Cells were washed three times with cold PBS and analyzed for surface expression of TLR4 using a FACSCalibur flow cytometer. To measure the surface expression of TNF-αR1, treated cells were labeled with unconjugated anti-human CD120a (TNF-αR1) polyclonal Ab (BD Biosciences) or relevant control Ab for 30 min at 4°C, washed twice with cold PBS, and incubated with FITC-conjugated goat anti-mouse secondary Ab (BD Biosciences). Cells were washed twice, resuspended in 1% paraformaldehyde solution, and analyzed on a FACSCalibur flow cytometer (BD Biosciences); data were analyzed using FACS Diva software.

**Real-time quantitative RT-PCR**

Total cellular RNA was extracted using an RNeasy Mini Kit (QiAGEN, Valencia, CA), and cDNA was synthesized from 1 μg of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). cDNA (50 ng) was amplified for real-time RT-PCR using Inventoried TaqMan Gene Expression Assay products (GAPDH: Hs03929097_g1, CCL2: Hs00234140_m1, TNF-α: Hs01113624_g1, CD68: Hs0238616_g1, IL-1β: Hs0155410_m1, TLR4: Hs00152939, CD163: Hs00174075_m1, IRF3: Hs01547283_m1), two gene-specific primers, one TaqMan MGB probe (6-FAM dye labeled), and TaqMan Gene Expression Master Mix in a 7500 Real-Time PCR System (Applied Biosystems). The mRNA levels were normalized against GAPDH mRNA, and the expression of CCL2 mRNA relative to control was calculated using the 2^-ΔΔCT method (20). Relative mRNA expression was shown as fold expression over the average of control gene expression taken as 1, and data are presented as mean ± SEM.

**Gene silencing**

Gene silencing was performed using the transient transfection method with an Amaxa Cell Line Nucleofector Kit V (Lonza) and Amaxa Electroporation System (Lonza), following the manufacturers’ instructions. For transient transfection, THP-1 cells (1 × 10⁶) were resuspended in Nucleofector solution and transfected separately using 30 nM IRF3 siRNA and scrambled negative control siRNA. After 36 h, transfected cells were treated with palmitate (200 μM) and TNF-α (10 ng/ml) for 24 h. Cells were harvested for RNA isolation, and conditioned media were collected for CCL2 production in supernatants. Immunoblotting analysis or real-time RT-PCR was also performed to assess the suppression of constitutive IRF3 or TLR4 expression in THP-1 cells transfected with IRF3 siRNA or TLR4 siRNA and scrambled negative control siRNAs.

**Measurement of NF-κB/AP-1 activity**

THP-1-XBlue cells are THP-1 cells that are stably transfected with a reporter construct, expressing SEAP gene under the control of a promoter inducible by NF-κB and AP-1 transcription factors. Upon stimulation, NF-κB and AP-1 are activated and lead to the secretion of SEAP in cell supernatant. THP-1-XBlue cells were stimulated with palmitate (200 μM) and TNF-α (10 ng/ml), alone or in combination, for 24 h at 37°C, and SEAP levels were detected in conditioned media after incubation for 4 h with QUANTI-Blue medium OD measurement at 650 nm wavelength.

**Western blotting**

THP-1 cells were harvested and incubated for 30 min with lysis buffer containing Tris (62.5 mM; pH 7.5), 1% Triton X-100, and 10% glycerol. Cell lysates were centrifuged at 14,000 rpm for 10 min, supernatants were collected, and protein was measured using Quick Start Bradford 1× Dye Reagent and a protein assay kit (Bio-Rad, Hercules, CA). Samples (20 μg) were mixed with loading buffer, heated for 5 min at 95°C, and resolved by 12% SDS-PAGE. Resolved proteins were transferred to an Immun-Blot PVDF Membrane (Bio-Rad) by electroblotting, blocked with 5% nonfat milk in PBS for 1 h, and incubated overnight at 4°C with primary Abs (1:100 dilution; Cell Signaling Technology) against IRF3 and p-IRF3. Blots were washed four times with TBS and incubated for 2 h with HRP-conjugated secondary Ab (Promega, Madison, WI); immunoreactive bands were developed using an Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, U.K.) and visualized using a Molecular Imager VersaDoc MP imaging system (Bio-Rad).

**Statistical analysis**

The data obtained were expressed as mean ± SEM, and group means were compared using an unpaired t test. The linear dependence or association between two variables was assessed by linear regression analysis. GraphPad Prism software (Version 6.05; La Jolla, CA) was used for statistical analysis, as well as for graphical representation of the data. All p values ≤ 0.05 were considered statistically significant.

**Results**

Palmitate and TNF-α act synergistically to trigger the production of CCL2 in monocytic cells

We asked whether palmitate and TNF-α exert a cooperative effect on CCL2 secretion in human monocytic cells. To this end, we

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FIGURE 1. Palmitate (PA) and TNF-α synergistically induce CCL2 in monocytic cells. (A) THP-1 cells were treated for 24 h with PA (200 μM), alone or in combination with TNF-α (10 ng/ml), before harvest. Total RNA was extracted, and Ccl2 mRNA was quantified by real-time PCR. Relative mRNA expression was expressed as fold change. (B) Secreted CCL2 protein in culture media was determined by ELISA. (C and D) Primary monocytes were isolated from PBMCs of healthy volunteers. Monocytes were incubated with PA and/or TNF-α for 24 h. Ccl2 mRNA (C) and secreted protein (D) were determined by real-time RT-PCR and ELISA. (E) THP-1 cells were immune-stained for confocal microscopy, as described in Materials and Methods. CCL2 expression is shown by green fluorescence (inset), whereas nuclei are stained blue with DAPI (original magnification ×40). (F) THP-1–derived macrophages were treated, as described above, and CCL2 protein expression was determined. (G and H) Migration assay was performed as described in Materials and Methods. Sample fluorescence (OD), expressed as RFU, was measured using excitation at 480 nm and emission at 520 nm. The number of cells migrating was calculated based on RFU values of test samples. (I) THP-1 cells were treated (Figure legend continues)
found that Ccl2 gene expression was significantly higher in THP-1 cells treated with palmitate and TNF-α compared with cells treated with palmitate or TNF-α alone (Fig. 1A). Similarly, CCL2 protein expression was also higher after palmitate and TNF-α cotreatment compared with either treatment alone (Fig. 1B). We stimulated THP-1 cells with different concentrations of TNF-α (0.5–20 ng/ml) and found that 10 ng/ml induced peak CCL2 concentrations in supernatants. Palmitate treatment (50–250 μM) also increased CCL2 production in a dose-dependent manner; we saw the best induction of synergy with a combination of 10 ng/ml TNF-α and 200 μM palmitate (Supplemental Fig. 1A, 1B). A similar synergistic relationship was observed between palmitate and TNF-α in primary human monocytes (Fig. 1C, 1D). This elevated CCL2 expression was confirmed by confocal microscopy (green fluorescence; Fig. 1E). Considering the functional significance of macrophages in adipose tissue inflammation, we further asked whether palmitate could also potentiate TNF-α–induced CCL2 production in macrophages. Using THP-1–derived macrophages, we found that the presence of palmitate enhances TNF-α–mediated production of CCL2 (Fig. 1F). Because CCL2 plays a central role in monocyte activation and migration, we used a Boyden chamber chemotaxis assay to test whether the CCL2 produced was biologically active. We found that conditioned media collected from monocyctic cells treated with palmitate and TNF-α induced a significantly higher chemotactic index (RFU; Fig. 1G) or higher cell migration (cell numbers; Fig. 1H) compared with controls.

Next, to determine whether synergy with TNF-α is specific to palmitate, we treated monocyctic cells with TNF-α and other long-chain fatty acids. We found that palmitate and oleate were most efficacious in this regard, whereas myristate had a smaller effect; laurate actually reduced the ability of TNF-α to induce CCL2 protein (Fig. 1I). Because the anti-inflammatory role of the short-chain fatty acid butyrate is well documented (21), we CCL2 protein was determined. All data are expressed as mean ± SEM (n ≥ 3). *p < 0.05, **p < 0.01, ***p < 0.001 versus PA or TNF-α alone.

Synergy between palmitate and TNF-α requires TLR4

Circulatory FFAs can serve as agonists of the innate immune receptor TLR4 (12). Therefore, we questioned whether our observed effects of palmitate require intact TLR4 signaling. We found that the treatment of cells with anti-TLR4 neutralizing Ab before costimulation with palmitate and TNF-α significantly suppressed the expression of CCL2 at the mRNA (Fig. 2A) and protein (Fig. 2B) levels. siRNA-mediated knockdown leads to the same conclusion, demonstrating that synergy between palmitate and TNF-α for CCL2 production requires TLR4 (Fig. 2C). Elevated levels of LPS, a known ligand for TLR4, are found in the circulation of obese individuals and have been linked with low-grade inflammation characterizing obesity or metabolic syndrome (22, 23), and LPS and TNF-α induce CCL2 in human brain endothelial cells (24). We hypothesized that LPS could substitute for palmitate in this cooperative production of CCL2, and this turned out to be the case (Fig. 2D, 2E). We also wanted to know whether this effect between palmitate and TNF-α involved modulation in the cognate receptor for TNF-α. In this regard, no significant change in TNF-R1 expression was observed after costimulation of cells with palmitate and TNF-α (Fig. 2F, 2G). We also examined the combined effect of IL-1β and palmitate on CCL2, because IL-1β is a strong activator of innate immune signaling and is elevated in the serum of obese individuals (25). No cooperativity was noted between IL-1β and palmitate with regard to CCL2 production (Fig. 2H, 2I).

Synergy between palmitate and TNF-α for production of CCL2 is independent of MyD88

TLR4 can mediate signaling through MyD88-dependent and MyD88-independent pathways to activate an inflammatory response. The MyD88-dependent pathway involves the production of proinflammatory cytokines/chemokines via the downstream activation of MAPK and NF-κB pathways (26). To determine whether the synergistic effect of palmitate with TNF-α requires MyD88, we used MyD88−/− monocytic cells with palmitate and TNF-α alone (Fig. 2J, 2K). In addition, inhibition of MAPK and NF-κB pathways by specific inhibitors significantly reduced the production of CCL2 in response to TNF-α/palmitate stimulation (Supplemental Fig. 1C–L).

Synergistic production of CCL2 by palmitate/TNF-α is TRIF dependent

In the MyD88-independent pathway of TLR4 signaling, the adaptor protein TRIF associates with TLR4 after clathrin-dependent internalization and leads to the activation of IRF3 and its downstream signaling cascade (27). To query whether this pathway was truly required for synergistic induction of CCL2 in monocyctic cells, we used chlorpromazine (CPZ) to inhibit clathrin-dependent endocytosis (28). Pretreatment with CPZ, followed by exposure to palmitate and TNF-α, caused a significant suppression of the synergistic production of CCL2 (Fig. 4A, 4B). Interestingly, the effects of TNF-α and palmitate alone were not altered by CPZ. Next, we confirmed the involvement of TRIF in this synergetic response using small molecule (resveratrolo) and peptide-based
(pepinh-TRIF) inhibitors. Pretreatment of monocytic cells with resveratrol abrogated the synergistic actions of palmitate and TNF-α (Fig. 4C, 4D). A similar effect was seen after blocking the TLR4–TRIF interaction with pepinh-TRIF (Fig. 4E, 4F).

Furthermore, the data show that increased NF-κB/AP-1 activity in monocytic cells after costimulation with palmitate and TNF-α was suppressed significantly by blocking endocytosis of TLR4 or by blocking TRIF activity (Fig. 4G, 4H).
IRF3 is required for the synergistic production of CCL2 by palmitate and TNF-α

To further verify that the synergistic induction of CCL2 by treatment of monocytic cells with palmitate and TNF-α was dependent on the TLR4-IRF3 signaling axis, we transfected cells with Irf3 siRNA, which achieved a ∼50% reduction in Irf3 mRNA levels compared with a scrambled siRNA control (Fig. 5A). Accordingly, the expression of Ccl2 mRNA and protein was significantly reduced in IRF3 siRNA-transfected cells after costimulation with palmitate and TNF-α (Fig. 5B, 5C). As with TRIF, disruption of IRF3 activity did not affect the ability of TNF-α or palmitate to induce Ccl2 mRNA or protein in isolation.

Because IRF3 is also activated by the TLR3 signaling pathway, we asked whether we could circumvent the need for TLR4 activation by specific activation of TLR3 by poly I:C (29). Our data show that poly I:C produced a synergistic effect with TNF-α that was comparable to palmitate, manifested by increased CCL2 expression (Fig. 5D, 5E) and phosphorylation of IRF3 (Fig. 5F, 5G). Altogether, our results show that IRF3 is a key effector of the synergistic actions of palmitate and TNF-α in monocytic cells.

Association between TNF-α and CCL2 in obese mice and obese humans

Our in vitro data show that palmitate and TNF-α synergistically upregulate CCL2. A number of previous studies reported increased circulatory levels of FFAs (30), TNF-α (16), and CCL2 (31) in HFD-induced obese (DIO) mice. Therefore, we hypothesized that increased circulatory CCL2 levels in DIO mice could result from elevated concentrations of TNF-α and FFAs. In our in vivo model study, mice were fed chow or an HFD for 16 wk. The data show significantly higher plasma levels of CCL2, TNF-α, and FFAs in HFD mice compared with chow-fed controls (Fig. 6A). As expected, our linear regression analysis data showed that plasma CCL2 levels were strongly correlated with TNF-α (r² = +0.84, p < 0.0001) and FFA (r² = +0.51, p = 0.014) levels in the circulation (Fig. 6B, Supplemental Fig. 2A). In addition, a significant association was found between plasma levels of TNF-α and FFAs in HFD mice (r² = +0.37, p = 0.048) (Supplemental Fig. 2B). Given these observations in the plasma, we next wanted to know whether similar changes were also occurring in WAT, which is a major driver of immunometabolic changes in obesity. Immunohistochemistry (IHC) shows significantly increased expression of CCL2 and TNF-α in the WAT of HFD mice compared with control mice. We also found a significantly higher presence of M1 (F4/80+) macrophages in WAT of HFD mice compared with control mice (Supplemental Fig. 2C). As expected, we found a significant correlation of CCL2 expression with TNF-α expression and macrophage marker F4/80 infiltration into adipose tissue as well (Fig. 6C, Supplemental Fig. 2D). Representative IHC images of these data showing increased expression of CCL2, TNF-α, and F4/80 macrophages in WAT of HFD mice and chow-fed mice are presented in Supplemental Fig. 2E and 2F. Taken together, these data suggest that obesity/FFAs favors increased circulatory and adipose tissue expression of CCL2.

The findings from our in vitro and mouse model studies led us to ask whether the observed association exists between CCL2 and

FIGURE 3. Synergistic induction of CCL2 by palmitate (PA)/TNF-α involves an MyD88-independent mechanism. (A and B) MyD88-knockout (KO) cells (THP1-XBlue-deMyD cells) were treated with PA (200 μm), 0.1% BSA (vehicle), or TNF-α (10 ng/ml), alone or in combination. Cells and culture media were collected after 24 h. CCL2 gene expression was determined by real-time PCR, and secreted CCL2 protein was determined in culture media by ELISA. (C) Cell culture media were also assayed for SEAP reporter activity representing the degree of NF-κB/AP-1 activation. All data are expressed as mean ± SEM (n ≥ 3). *p < 0.05, **p < 0.01, ***p < 0.001 versus PA or TNF-α alone.
TNF-α in s.c. fat samples from obese subjects compared with lean and OW individuals. In fat tissue, Ccl2 and Tnf-α mRNA levels were high in obese subjects compared with lean/OW individuals (Fig. 6D, 6E). Adipose tissue Ccl2 mRNA expression was positively correlated with body mass index (BMI) and percent body fat (Supplemental Fig. 3A, 3B). Interestingly, in obese adipose tissue, our linear regression analysis showed a positive correlation between Ccl2 and Tnf-α mRNA expression ($r^2 = +0.21$, $p = 0.023$) (Fig. 6F), whereas no such association existed in lean/OW individuals (Supplemental Fig. 3C). Consistent with the mRNA levels, the protein expression of CCL2 and TNF-α was high (Supplemental Fig. 3D, 3E) in obese adipose tissue and exhibited a mutual positive association ($r^2 = +0.47$, $p = 0.0007$) (Fig. 6G). Elevated levels of Ccl2 mRNA expression in adipose tissue correlated with the macrophage marker CD68 in obese individuals (Supplemental Fig. 3F, 3G) but not in lean/OW subjects (Supplemental Fig. 3H). A strong colocalization of CCL2 and TNF-α expression was observed by confocal microscopy in obese human tissues compared with lean samples (Supplemental Fig. 3I, 3J). Similarly, strong IHC staining was observed for CCL2 and
TNF-α in obese samples compared with lean fat samples (Supplemental Fig. 3K, 3L). Overall, CCL2 and TNF-α show an elevated and strongly associated expression in human obese adipose tissue.

To summarize the underlying signaling pathways involved in this synergistic interaction between palmitate and TNF-α for CCL2 production, a schematic illustration is presented in Fig. 7.

**Discussion**

A large number of studies implicate proinflammatory macrophage recruitment into adipose tissue as a key driver of the low-grade inflammation and insulin resistance commonly associated with obesity (1, 2). CCL2 (MCP-1) is a major factor promoting monocyte recruitment from the circulation into adipose tissue during overnutrition (32, 33). CCL2 levels are very high in WAT.
and plasma in obesity (3, 31, 32, 34–36). However, the mechanisms by which CCL2 becomes upregulated in obesity remain elusive. In this study, we report that FFAs, including palmitate, potentiate the TNF-α–mediated production of CCL2 in monocytic cells and macrophages. Our results demonstrate that the long-chain fatty acids palmitate, oleate, and myristate synergistically or additively enhance CCL2 production and secretion by monocytic cells when combined with TNF-α. Interestingly, the medium-chain fatty acid laurate and the short-chain fatty acid butyrate did not show this cooperative effect with TNF-α.

Many studies have reported that CCL2 can be produced in vitro by various cell types, primarily monocytes/macrophages and adipocytes, in response to different stimuli, including TNF-α (7), FFAs (9, 37), and LPS (6, 9, 10). Notably, no previous study has elaborated on the synergistic effect between FFAs and TNF-α for CCL2 production. Substantial progress has been made in understanding the ability of FFAs to activate TLR4 signaling in monocytes/macrophages and adipocytes (37–40). Therefore, it was important to determine whether TLR4-mediated signaling is involved in the synergy between palmitate and TNF-α. In this study, we present three forms of evidence that the synergistic effect between palmitate and TNF-α depends on TLR4. First, we observed that synergistic production of CCL2 by palmitate and TNF-α is suppressed by Ab-mediated neutralization of TLR4. Second, TLR4-deficient cells do not support synergistic secretion of CCL2 in response to stimulation with palmitate and TNF-α. Third, we showed that palmitate, like LPS, can interact with TLR4 and initiate signals that activate NF-κB, JNK, ERK1/2, MAPKs, and AP-1, all of which are known to participate in the development of inflammation and insulin resistance (38, 40–46). Of note, TLR4-deficient mice fed an HFD had decreased circulating CCL2 and lower macrophage infiltration into adipose tissue (38). In metabolic inflammation induced by obesity, saturated FFAs may act as TLR4 ligands and cause induction of TNF-α in monocytes/macrophages, underscoring potential pathological interactions.

**FIGURE 6.** Elevated CCL2 is correlated with TNF-α in DIO mice and obese humans. (A) Male mice were fed an HFD (n = 11) or chow (n = 9) for 16 wk. Plasma levels of TNF-α, CCL2, and FFAs were determined. (B) Correlation between plasma levels of CCL2 and TNF-α in obese mice. (C) WAT samples were obtained from DIO mice (n = 7). Adipose tissue CCL2 and TNF-α protein expression was determined by IHC. Staining intensity is shown in arbitrary units (AU). CCL2 protein expression correlated positively with TNF-α protein expression in the adipose tissue of obese mice. (D and E) Sections of s.c. adipose tissue from lean/OW (n = 26) and obese (n = 26) individuals. Increased adipose tissue expression of mRNA of Ccl2 and Tnf-α was detected by real-time RT-PCR and represented as fold change over controls. Each dot represents an individual value for Ccl2 or Tnf-a mRNA. Horizontal lines represent the mean values of adipose tissue CCL2 and TNF-α in each group. (F) Correlation of Ccl2 mRNA with Tnf-α mRNA in obese individuals. (G) CCL2 and TNF-α protein expression was detected by IHC. Protein expression was represented as staining intensity based on Aperio-positive pixel counts (Aperio software algorithm version 9.0). Correlation of CCL2 protein levels with TNF-α protein in obese individuals. *p < 0.05, **p < 0.01 versus controls.
FIGURE 7. Schematic illustration of signaling pathways underlying the synergy between palmitate and TNF-α for CCL2 production. The pathway highlighted with red arrows represents the predominant mechanism of this synergistic response induced by palmitate. CPZ may inhibit TLR4 internalization and suppress synergy between palmitate/TNF-α for production of CCL2.

between FFAs and TLR4 (12, 47). In mice, endotoxemia due to the intake of dietary fats has been shown to cause inflammation via a TLR4-mediated signaling mechanism (22), supporting a key role for TLR4 in linking consumption of dietary fats with metabolic inflammation and insulin resistance. Our data indicate that palmitate mimics the effect of LPS in triggering TNF-α-mediated CCL2 production. Our data further show enhanced activation of NF-κB/AP-1 in response to combined treatment with palmitate/LPS and TNF-α. TLR4 and TNF-α agonistic responses are known to activate NF-κB/AP-1 classical signaling pathways in mononuclear cells (40, 48, 49). Thus, multiple lines of evidence point to the conclusion that FFAs may initiate inflammatory signaling, at least in part, by activating TLR4 signaling in monocytic cells.

TLR4-mediated signaling activates two major downstream signaling pathways: the MyD88-dependent and MyD88-independent (TRIF/IRF3-dependent) pathways. MyD88-dependent signaling pathways involve interaction between IRAK1 and TRAF6, leading to activation of NF-κB and the MAPKs JNK and transcription factor AP-1. The MyD88-independent (TRIF/IRF3) pathway involves activation of downstream molecules, including TANK-binding kinase 1 and inhibitor of IκB kinase ε, which, in turn, phosphorylate and activate IRF3 (50–52). To broaden our understanding of TLR4 signaling in the synergy between palmitate and TNF-α in inducing CCL2 secretion, we first focused our attention on MyD88. Specifically, we demonstrated that synergistic CCL2 production was not affected by the absence of MyD88. However, the individual effect of palmitate on CCL2 production was completely abolished. We (40) and other investigators (46, 53–55) showed that palmitate triggers the activation of NF-κB/AP-1 via the MyD88-dependent pathway for production of inflammatory mediators; our current results demonstrate that synergistic activation of NF-κB/AP-1 by palmitate and TNF-α is independent of MyD88. This led us to speculate that the TRIF/IRF3 signaling pathway downstream of TLR4 may act as a regulator of this synergy. The role of TRIF/IRF3 signaling in this synergistic effect between palmitate and TNF-α was confirmed through four approaches. First, we show that blocking ligand-induced TLR4 internalization suppresses synergy between palmitate and TNF-α. Our results are in line with previous studies showing that inhibition of clathrin-dependent TLR4 endocytosis abolishes the LPS-induced expression of TRIF-dependent genes, including IFN-β, RANTES (CCL5), and IL-6 (56–58). Collectively, these data suggest that clathrin-dependent endocytosis of TLR4 plays a critical role in the expression of TRIF target genes, including the synergistic effect of palmitate and TNF-α on CCL2. Second, using pharmacological and molecular inhibitors of TLR4/TRIF complex formation, we found that TRIF involvement was critical to synergy between palmitate and TNF-α for CCL2 production. Blocking of TRIF/TLR4 complex formation suppresses LPS-induced cytokine production (59). Third, we show that poly I:C-mediated phosphorylation/activation of IRF3 mimics the effect of palmitate in the cooperative actions on CCL2 secretion. We also show that palmitate leads to phosphorylation of IRF3, like other upstream activators, such as poly I:C or LPS (60). Fourth, IRF3-deficient cells failed to respond synergistically to palmitate and TNF-α costimulation. In parallel, NF-κB/AP-1 activation was significantly reduced in IRF3-deficient cells. TLR3/TLR4 were shown to activate NF-κB/MAPKs in MyD88-deficient cells (61). Similarly, IRF3 activation by various receptors, along with activation of NF-κB and MAPKs, led to the activation of target genes, including IP-10 (CXCL10), RANTES (CCL5), IFN-stimulated gene 56 (also known as IFN-induced protein with tetratricopeptide repeats 1), and arginase II (62). TRIF activation by LPS/TLR4 signaling led to activation of the IRF-3/IRF-7- and NF-κB-dependent signaling pathways (63, 64). To sum up the molecular mechanisms of this synergy between palmitate and TNF-α for the production of CCL2, we suggest that the TRIF/TRAM/IRF3 signaling axis is predominantly involved in this cooperative response, because MyD88-competent and MyD88-null cells showed comparable activity.

Our data showing that plasma levels of CCL2, TNF-α, and FFAs are elevated in the circulation of obese mice is consistent with prior reports (3, 16, 30). Our data also point to a profound relationship among these immune-metabolic markers in obesity. In the adipose tissue samples from DIO and control mice, our data show high expression of CCL2 and TNF-α, together with increased infiltration of activated macrophages. In addition, we note a strong association between CCL2 and both TNF-α and adipose tissue F4/80 macrophages in obese mice. CCL2 (3), TNF-α (16), and FFAs (11) have been shown to orchestrate macrophage accumulation and inflammation in adipose and other tissues; we speculate that simultaneously elevated plasma levels of these three crucial markers may have the potential to induce significant pathophysiological changes in the adipose tissue compartment in obesity, including changes in insulin sensitivity and lipid metabolism.

As in mice, our results reveal that human adipose tissue shows high expression of CCL2 and TNF-α, along with a monocyte/macrophage marker (CD68), in obese subjects compared with lean/OW controls. In addition, CCL2 expression in human adipose tissue positively correlated with TNF-α and inflammatory macrophage markers, whereas no such association was found in adipose tissue samples from lean or OW individuals. Adipose tissue of humans exhibits increased expression of TNF-α (15, 65), IL-6 (66), FFAs (11), and CCL2 (32) compared with lean controls. Moreover, our data show a positive correlation between CCL2 and BMI. The abundance of Ccl2 mRNA in s.c. adipose tissue was found to correlate significantly with BMI (65, 67). These observations are consistent with the argument that TNF-α,
under the influence of FFAs, enhances CCL2 production in obesity, contributing to macrophage recruitment to adipose tissue and to the development of metabolic inflammation generally. Obesity induces dysregulation in the adipose tissue, leading to increased expression of adipokines, such as TNF-α, IL-6, and CCL2, which inflict damage to adipocytes, a source of FFAs (2, 68), and the combined interaction between TNF-α and FFAs may trigger further CCL2 production. In the adipose tissue, immune cells and adipocytes express receptors for TNF-α and FFAs, a mechanism that supports a cross-talk model in which adipocytes and immune cells act as the source, as well as the target, of proinflammatory signals.

In conclusion, our results show that there is a synergistic interaction between TNF-α and palmitate that depends on TLR4/IRF3 and leads to the elevation of CCL2, providing interesting pathophysiologic connections among FFAs, TNF-α, and CCL2 in settings such as obesity.

Acknowledgments

We thank Dr. Fahad Al-Ghamlas for help with patient recruitment.

Disclosures

The authors have no financial conflicts of interest.

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